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The folate antagonist, methotrexate, is a potent inhibitor of murine and human cytomegalovirus in vitro

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Summary

Cytomegalovirus (CMV) is a major source of morbidity for immunocompromised patients, such as AIDS patients. The folic acid antagonists have not been explored as potential antiviral agents against CMV. We examined the effects of methotrexate, compared to acyclovir and ganciclovir, on both murine CMV (MCMV) and human CMV (HCMV) in vitro. Using a plaque assay in mouse embryo cells or human foreskin fibroblasts for MCMV and HCMV respectively, we found that methotrexate, in micromolar concentrations, was a potent inhibitor of both viruses. This effect was due to folic acid antagonism since folinic acid abrogated the antiviral effect of methotrexate, but not ganciclovir. Cellular toxicity due to methotrexate appeared insufficient to account for the antiviral effects. The ability of methotrexate to inhibit CMV in vivo merits exploration.

Methotrexate; Human cytomegalovirus; Murine cytomegalovirus

Introduction

Cytomegaloviruses, which cause infections in a variety of vertebrates including man, are members of the Herpesviridae (Weller, 1971). In humans, cytomegalovirus (CMV) infections are common, but diseases due to CMV are largely confined to infections in utero and infections of individuals with abnormalities of im-

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mune defenses (Betts, 1977). Because of the rising incidence of serious CMV infections in individuals with abnormal host defenses, there is a search for agents with antiviral activity against this virus.

Very little information is available concerning the effect of folate antagonist drugs on CMV replication in vitro or in vivo. We have examined the effects of methotrexate, an inhibitor of dihydrofolate reductase, on the in vitro replication of both murine CMV (MCMV) and human CMV (HCMV). Our results indicate that methotrexate is a potent inhibitor of the replication of both viruses.

Methods

Reagents

Methotrexate, thymidine and folinic acid were purchased from Sigma Chemical Company (St. Louis, MO). Ganciclovir was provided by Syntex Corp. (Palo Alto, CA) and acyclovir was provided by Burroughs Wellcome Corp. (Research Triangle, NC). [^{3}H]thymidine (84 mCi/mol) and [^{3}H]uridine (40.2 mCi/mol) were purchased from New England Nuclear (Boston, MA).

Cell culture

Mouse embryo cells (MEC) were prepared from the embryos of late term pregnant CD-1 mice by trypsin-EDTA desegregations. Human foreskin fibroblast cells (HFF) were prepared from newborn foreskins by collagenase treatment. MEC and HFF were maintained as monolayers in 75 cm^2 tissue culture flasks using Eagle's minimum essential medium (MEM) containing 10% fetal calf serum (FCS), 100 units/ml penicillin, and 50 μg/ml gentamicin.

Viruses

Murine cytomegalovirus (MCMV), obtained from M.C. Jordan (University of Minnesota, Minneapolis, MN), has been maintained by serial passage in BALB/c mice. Virus inoculum was prepared by inoculating mice intraperitoneally with 1×10^5 plaque forming units (PFU) of MCMV in 0.2 ml phosphate buffered saline (PBS). Seventeen days after infection, the mice were killed and the salivary glands prepared as a 10% (w/v) homogenate in MEM, 10% dimethylsulfoxide (DMSO), 10% FCS, and antibiotics. The stock virus, which contained 1.5×10^7 PFU/ml MCMV, was stored at −70°C.

The AD 169 strain of human cytomegalovirus (HCMV) was obtained from American Type Culture Collection (Bethesda, MD). Stock virus was prepared by infecting monolayers of HFF in 75 cm^2 tissue culture flasks at a multiplicity of infection of 0.1. Seven to ten days after inoculation, when viral cytopathic effects involved 100% of the cells, the culture supernatants were collected, clarified by low speed centrifugation, and stored at −70°C.

Plaque reduction assays

Quantitation of MCMV and HCMV was performed by plaque assay in MEC (24 well tissue culture plates) or HFF (25 cm^2 flasks) monolayers respectively, using

an overlay of MEM 10% FCS, 0.02 M HEPES, antibiotics, and 1% methylcellulose.

Assay of compounds for antiviral activity against MCMV was performed by plaque reduction using MEC monolayers prepared in 24-well tissue culture plates. Monolayers were inoculated with 50 PFU MCMV in 0.1 ml of MEM with 10% FCS, and antibiotics and adsorbed for 1 h at 37°C. The monolayers were covered with 2 ml MEM, 10% FCS, 0.2 M HEPES, and 1% methylcellulose and various concentrations of drug. After 5 days, the cells were fixed with ethanol, glacial acetic acid, and formalin (6:2:1) (EAF) and stained with 1% crystal violet.

Assay against HCMV was performed in HFF monolayers grown in 25 cm^2 flasks. Monolayers were inoculated with 200 PFU HCMV in 0.5 ml medium for 1 h at 37°C, then overlaid with 5 ml 1% methylcellulose solution containing various concentrations of drug. After 12 days the monolayers were fixed with EAF and stained with 1% crystal violet. For both cell types, assays were conducted twice and each individual sample performed in triplicate. The number of plaques were plotted as a function of drug concentration and the concentration of drug producing 50% reduction in plaque formation, the effective dose 50 (i.e. 1 ED_{50}), was determined.

Drug cytotoxicity

The effect of the various compounds on a variety of cell functions was assessed. 1×10^5 MEC or HFF in MEM containing 10% FCS, and antibiotics were seeded into 6-well tissue culture plates. Two hours after seeding, the cells were exposed to either no drug, or 0.1, 1.0, and 10 ED_{50} of the drug to be tested. In addition, 5 μCi of either [^{3}H]thymidine or [^{3}H]uridine was added to a number of wells. Four and 96 h after placement of the cells, the cultures were assessed for cell proliferation, protein content, and incorporation of ^{3}H into DNA and RNA. The cells from three wells were pooled after being mobilized by trypsin. Cells were washed twice in PBS, and suspended in PBS at a final volume of 1.0 ml. Cell number was determined by counting the cells in a hemocytometer. The protein content was determined by Lowry assay, using lysozyme as a standard (Lowry et al., 1951). The synthesis of RNA and DNA was assessed by lysing the cells with 1% sodium dodecylsulfate. Twenty microliters of cell lysate was then spotted on a Whatman paper disc, which was placed in 5% trichloroacetic acid (TCA). The incorporation of [^{3}H]uridine or [^{3}H]thymidine radiolabel into TCA precipitable counts was measured using a liquid scintillation counter. All determinations were performed in triplicate.

Results

The antiviral effect of methotrexate

The effects of methotrexate on both MCMV and HCMV replication were compared to acyclovir and ganciclovir, using a plaque reduction assay (Table 1). Methotrexate produced a significant dose-related reduction in MCMV replication, which was comparable to both acyclovir and ganciclovir. Similarly, methotrexate

TABLE 1

The concentrations (μM) of methotrexate, acyclovir, and ganciclovir producing 50% inhibition of HCMV and MCMV plaque formation (ED_{50}) by stock virus pools in human foreskin fibroblasts and murine embryo cells respectively

Drug	Drug concentration (μM) producing 50% plaque reduction	
	MCMV	HCMV
Acyclovir	0.89	ND
Ganciclovir	2.35	1.37
Methotrexate	0.22	0.06

ND, not done.

was effective in inhibiting HCMV replication and was substantially more effective than ganciclovir.

To determine if the antiviral effect of methotrexate was due to inhibition of dihydrofolate reductase, MCMV and HCMV replication was examined in the presence of 1 ED_{50} of methotrexate or ganciclovir and various concentrations of folinic acid (Table 2). Folinic acid abrogated the antiviral effect of methotrexate against both MCMV and HCMV, but had no significant effect on the antiviral activity of ganciclovir.

Since methotrexate can directly effect thymidylate synthetase (Huennekens et al., 1987), we examined the ability of excess thymidine to prevent the antiviral effects of methotrexate. Thymidine at 1.0, 10, and 100 μg/ml had no effect on the ability of 1 ED_{50} methotrexate to inhibit HCMV replication (data not shown). Thus, the antiviral activity of methotrexate appears to be mediated by inhibition of dihydrofolate reductase, but not thymidylate synthetase.

TABLE 2

The effects of various concentrations of folinic acid on the inhibition of HCMV and MCMV plaque formation by 1 ED_{50} methotrexate or ganciclovir

Drug	Virus titer (PFU/ml)[a] Folinic acid concentration[b]			
	0	1	10	100
A. HCMV				
none	112	–	–	–
methotrexate (0.06 μM)	33	ND	112	125
ganciclovir (0.3 μM)	51	ND	48	79
B. MCMV				
none	95	–	–	–
methotrexate (2.2 μM)	10	21	80	74
ganciclovir (1.4 μM)	45	60	57	40

[a]Monolayers of MEC or HFF in 25 cm^2 flasks were inoculated with approximately 100 PFU of MCMV or HCMV. Following inoculation, cells were exposed to 1 ED_{50} methotrexate and various concentrations of folinic acid.

[b]Concentration of folinic acid (μg/ml).

ND, not done.

Methotrexate cytotoxicity for MEC and HFF

Methotrexate has significant effects on the metabolic activities of dividing cells, acting at a variety of sites. At high concentrations, methotrexate is toxic to metabolically active cells (Goldman, 1977; Jackson and Grindey, 1985; Jackson, 1987). Therefore, we examined MEC and HFF cells for evidence of methotrexate induced cytotoxicity. At concentrations of methotrexate above 20 μM, both HFF and MEC monolayers exhibited morphological changes indicating cell toxicity. However, at 1 ED_{50} (0.22 μM for MCMV and 0.06 μM for HCMV), no morphological changes indicating cellular toxicity were evident in either MEC or HFF.

Infection of cells by both MCMV and HCMV appears to stimulate a number of cellular metabolic functions, and there is evidence that both viruses may depend on these metabolic functions for effective viral replication (Rapp, 1983; Griffiths and Grundy, 1987). Therefore, we examined the effects of methotrexate on cellular proliferation, protein synthesis, and the incorporation of [^{3}H]uridine and [^{3}H]thymidine into RNA and DNA, respectively. In MEC, 0.1, 1.0 and 10 MCMV ED_{50} of methotrexate suppressed, but did not eliminate, protein synthesis and incorporation of [^{3}H]uridine (Table 3). Cell proliferation was reduced at 0.1 ED_{50} and completely eliminated at 1 and 10 ED_{50}. The incorporation of [^{3}H]thymidine into DNA was not affected at 0.1 and 1 ED_{50}, but was increased at 10 ED_{50}. Comparable effects of methotrexate on cell proliferation, [^{3}H]uridine and [^{3}H]thymidine incorporation, and protein synthesis were seen in HFF (data not shown). As with MEC, the incorporation of [^{3}H]thymidine into DNA was significantly increased in HFF in the presence of 10 ED_{50} (0.6 μM) of methotrexate. This was presumably due to inhibition of thymidylate synthetase, and preferential utilization of the thymidine kinase (TK) salvage pathway. Since TK is a periodic enzyme, these data would suggest that cellular activities can still be induced in the presence of methotrexate (Blakley and Vitols, 1968).

TABLE 3

The effects of methotrexate (0.1, 1.0, and 10 ED_{50}) or ganciclovir (1.0 and 10 ED_{50}) on mouse embryo cell (MEC) proliferation, [^{3}H]thymidine and [^{3}H]uridine[a] incorporation over a four day period

Treatment	ED_{50}	Cell count ($\times 10^4$)		[^{3}H]thymidine incorporation[b] (CPM)		[^{3}H]uridine incorporation[b] (CPM)	
		D0	D4	D0	D4	D0	D4
No drug		27	127	517	21080	214	6139
Methotrexate	0.1[c]	–	44	–	17801	–	4294
	1.0	–	23	–	20694	–	3614
	10	–	25	–	33587	–	2722
Ganciclovir	1.0[c]	–	160	–	ND	–	6310
	10	–	65	–	11337	–	6031

[a]Measured by radiolabelled nucleoside incorporation into TCA precipitable counts.
[b]Measured as counts per minute per 20 μl cell lysate.
[c]The dose of drug producing 50% inhibition of MCMV (i.e. 1 ED_{50}) in MEC was 0.22 and 2.4 μM for methotrexate or ganciclovir, respectively.

TABLE 4

The effect of methotrexate (0.1, 1.0 and 10 ED_{50}) or ganciclovir (1.0 and 10 ED_{50}) on human foreskin fibroblast (HFF) proliferation, [^{3}H]thymidine and [^{3}H]uridine[a] uptake over a four day period

Treatment	ED_{50}	Cell count ($\times 10^4$)		[^{3}H]thymidine incorporation[b] (CPM)		[^{3}H]uridine incorporation[b] (CPM)	
		D0	D4	D0	D4	D0	D4
No drug		10	30	67	6455	43	975
Methotrexate	0.1[c]	–	15	–	8596	–	1117
	1.0	–	15	–	8530	–	1009
	10	–	12	–	17484	–	820
Ganciclovir	1.0[c]	–	23	–	10225	–	1440
	10	–	29	–	9974	–	1226

[a]Measured by radiolabelled nucleoside incorporation into TCA precipitable counts.
[b]Measured as counts per minute per 20 μl cell lysate.
[c]The dose of drug producing 50% inhibition of HCMV (i.e. 1 ED_{50}) in HFF was 0.06 and 1.4 μM for methotrexate or ganciclovir, respectively.

Discussion

Methotrexate is an antimetabolite which blocks the activity of dihydrofolate reductase, an enzyme which catalyzes the conversion of dihydrofolic acid to tetrahydrofolic acid (Jackson and Grindey, 1985). The latter compound is important in the synthesis of nucleic acids, affecting the de novo synthesis of purines and the conversion of uridine monophosphate to thymidine monophosphate. In addition, methotrexate can affect thymidylate synthetase and serine hydroxymethyl transferase. As such, methotrexate can affect cellular DNA and RNA metabolism, as well as protein synthesis. At high concentrations, methotrexate is toxic to metabolically active cells.

Little attention has been paid to the antiviral effects of folic acid antagonists, such as methotrexate, on cytomegalovirus replication. Our data indicate that methotrexate is a potent inhibitor of both MCMV and HCMV replication in vitro. These studies indicate that methotrexate is active at concentrations comparable to those of both acyclovir and ganciclovir. The antiviral effect of methotrexate is mediated by inhibition of dihydrofolate reductase, since its antiviral effects are abolished by folinic acid.

Although our studies indicate that methotrexate is a potent inhibitor of HCMV and MCMV via its action on dihydrofolate reductase, its precise mechanism of antiviral activity remains undefined. Methotrexate is known to inhibit DNA synthesis by inhibition of thymidylate synthetase and by inhibition of purine synthesis. Although the inhibition of DNA synthesis in MEC and HFF cells was not absolute, it may well be that the reduction of DNA synthesis was sufficient to suppress CMV replication. Alternatively methotrexate may inhibit a cellular function needed by CMV to replicate. During viral infection, the cytomegaloviruses are known to stimulate cellular metabolism, and appear to depend on a number of cellular func-

tions for effective virus replication (Rapp, 1983; Griffiths and Grundy, 1987). While the precise functions are unknown, they appear to require protein synthesis. Methotrexate, by virtue of its effect on purine synthesis, also affects RNA metabolism, and ultimately, protein synthesis. Thus it is possible that methotrexate acts by preventing the induction of cellular functions which are needed for CMV replication. It is interesting, however, that the [^{3}H]thymidine incorporation data for HFF indicated that the thymidine kinase (TK) pathway was operative in the presence of 10 ED_{50} methotrexate. TK, the enzyme in this periodic salvage pathway, is generally seen in cells only after induction (Blakley and Vitols, 1968). Its presence indicates that the doses of methotrexate which are effective in inhibiting virus replication were not sufficient to prevent the induction of thymidine kinase. Thus, further work will be needed to determine the antiviral mechanism of methotrexate.

Methotrexate has been used as an antimetabolite in clinical practice for over two decades (Chabner et al., 1975). Although it is potentially toxic in high doses or prolonged administration, judicious use of this agent has been useful in a number of disease states. Doses of methotrexate of 25 to 100 mg/m^2 generally produce blood levels of 1 to 100 μM, 60 times the in vitro ED_{50} for HCMV (Calabresi and Parks, 1985). However, toxicity is known to develop with prolonged methotrexate levels of 0.1 μM, 6 times the ED_{50}. The clinical utility of methotrexate in CMV infections in vivo is not known. Although toxicity may limit its clinical utility in vivo, the potential of methotrexate as an antiviral agent for CMV infections merits further investigation.

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